A Method to Monitor DNA Transfer During Transfection

Submitted: January 21, 1999; Accepted: June 21, 1999; Published: June 25, 1999.

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ABSTRACT This report describes a method for monitoring the transfer of DNA during transfection. This method involves random labeling of plasmid DNA with fluorescein-12-dUTP, flow cytometric detection and sorting of the fluorescent transfected cells, and laser confocal fluorescence microscopic determination of the intracellular location of the plasmid DNA. By this method, >95% of the sorted cells were labeled, indicating a >95% specificity of the sorting procedure. The sorted cells were viable, as indicated by their ability to exclude trypan blue dye (>98% cells excluded the dye) and to maintain cell growth. The results of the kinetics of the Lipofectin transfection technology show that the fraction of the cells that internalized plasmid DNA increased from 10% at 1 hr after initiation of the transfection procedures to 18% at 3 hr. This method does not require protein expression, does not require the use of selection pressure such as drug treatment to isolate the cells that internalized DNA, and can be used to study the early events of DNA transfection.

KEY WORDS: transfection, cell sorting, fluorescein-labeled DNA, lipofectin, confocal microscopy

INTRODUCTION

Introduction of exogenous DNA into cells has been an important tool for studying the structural and functional biology of the cell. The commonly used DNA transfection procedures are (a) chemical methods, including calcium phosphate precipitation, DEAE-dextran complexation and lipid-mediated DNA transfer; (b) physical methods, including electroporation, microinjection, and biolistic particle delivery; and (c) recombinant viral vectors (1,2). The majority of previous investigations have focused on elucidating the function of the transfected genes. Relatively little is known about the kinetics of transfection or on the fate of the transfected DNA, such as the transfer of DNA from the extracellular to the intracellular space and intracellular transfer and localization. This is in part because the conventional methods for monitoring gene transfer use the expression of reporter genes (eg, luciferase, green phosphorescent protein, resistance to neomycin and chloramphenicol (3-10)), which require 24 to 48 hr for gene transcription and translation and are not suitable for studying the early or intermediate events during transfection.

Two recent studies examined the fate of DNA during transfection with cationic lipids. One study used DNA labeled with the fluorescent ethidium monoazide and flow cytometry to determine the kinetics of transfection (ie, changes in the fraction of cells that incorporated the DNA) and used gold-labeled DNA and electron microscopy to determine the intracellular location of the transfected DNA. The results show that the fraction of transfected cells increased slowly with time during transfection, that the transfected DNA resided in a perinuclear location, and that most cells contained at least some of the transfected DNA in the cytoplasm after transfection (11). The second study used ethidium-labeled DNA to determine the rate of plasmid uptake in relation to the expression of a reporter gene; the results show that more than 95% of cells contained the transfected DNA after 3 hr of transfection, whereas the gene expression was delayed, reaching a maximum level after 48 hr (12). Both studies found a correlation between the amount of transfected DNA in the cells and the extent of gene expression, which suggests the entry of the transfected DNA into the nucleus. However, because the labeled DNA was not detected in the nucleus, which is assumed to be due to the low level of the transfected DNA in the nucleus (11), there

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remain unanswered questions regarding both the kinetics of cytoplasm-to-nucleus transfer of the transfected DNA and the kinetics of gene expression after DNA enters the nucleus. In order to address these questions, additional methodologies that can detect low levels of DNA transfer in intracellular compartments, especially during the earlier times before gene expression takes place, are needed.

This article describes a method for monitoring the kinetics of the transfer of exogenous DNA during transfection. It involves random labeling of plasmid DNA with fluorescein-12-dUTP, flow cytometric detection and sorting of the fluorescent transfected cells, and laser confocal fluorescence microscopic determination of the intracellular location of the plasmid DNA. This method could detect cells containing internalized DNA as early as 1 hr after transfection and provide the intracellular location of the transferred DNA.

MATERIALS AND METHODS

Plasmid Preparation

The pGL3-control plasmid (Promega, Madison, WI) was purified from JM109 Escherichia coli cells (Promega) using the Qiagen maxi kit (Qiagen, Santa Clarita, CA). Plasmid DNA was quantified by UV absorbance at 260 nm on a spectrophotometer (Beckman DU640, Fullerton, CA). Nonspecific labeling of the plasmid with fluorescein-12-dUTP was performed using a commercially available nick translation kit (Boehringer Mannheim, Indianapolis, IN). Briefly, 100 µl of a solution containing fluorescein-12-dUTP (4.2 nM) and dNTPs (33.3 µM dTTP, 100 µM dATP, 100 µM dGTP, 100 µM dCTP) was mixed with 100 µl of nick translation buffer (2× reaction buffer, 0.2 volumes DNA Pol-I enzyme, and 100 µg plasmid DNA). The average molar ratio of fluorescein-12-dUTP to plasmid was 143. The mixture was incubated at 15oC. The reaction was stopped by incubation in a water bath at 65oC for 10 min. The plasmid DNA was then precipitated with 3 volumes of 100% ethanol. After centrifugation at 19,000g for 30 min, the resulting pellet was washed once with 70% ethanol and allowed to air dry for 15 min and then resuspended in 1 ×TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA).

The relationship between the size of the plasmid DNA and the duration of the nick translation procedures was evaluated. DNA was analyzed by electrophoresis using a 1% agarose gel. DNA was visualized by ethidium bromide staining. The fluorescence label was visualized using a UV transilluminator (Ultra-Lum, Carson, CA).

Cell Culture and Transfection

Human head and neck FaDu squamous carcinoma cells (American Type Culture Collection, Rockville, MD) were cultured in a humidified atmosphere containing 95% air and 5% CO2 at 37oC. The cell culture media consisted of Minimal Essential Medium containing Earl's salts supplemented with 9% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 100 µg/ml gentamicin, 100 µg/ml cefotaxime sodium and 1.8 mM L-glutamine (Gibco-BRL, Grand Island, NY). Cells were maintained at subconfluent conditions in exponential growth phase. Cells were harvested with 0.05% trypsin and versene (0.1 M NaCl,1.5 mM KH2PO4, 2.7 mM KCl, 8.0 mM Na2HPO4, 1.0 mM EDTA, 5.6 mM glucose), and cell number was counted with a Bright-Line (Hausser Scientific, Horsham, PA) hemocytometer.

For transfection, cells were plated at a density of 5 × 105 cells per 10 ml in 100 mm diameter tissue culture treated petri dishes (Corning, Corning, NY) and allowed to attach overnight. Transfection was performed using Lipofectin (Gibco-BRL). Lipofectin was incubated with serum free media (Opti-Mem, Gibco-BRL), at a ratio of 5 to 100 µl, at room temperature for 45 min. One hundred microliters of the premixed Lipofectin/Opti-Mem was incubated with 4 µg plasmid and 100 µl Opti-Mem for 15 min at room temperature. After incubation, the plasmid/Lipofectin/Opti-Mem mixture was brought up to a final volume of 1 ml using Opti-Mem medium. The final mixture was added drop-wise to cells. At predetermined time points, the plasmid-containing medium was removed and cells were washed sequentially with 10 and 5 ml of versene. Cells were then incubated with 1 ml of 0.05% trypsin for 10 min, followed by the addition of 8 ml of serum enriched medium. The resulting cell suspension was centrifuged and resuspended in 1 ml of Hank's Balanced Salt Solution (HBSS, Gibco-BRL)

supplemented with 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) and sorted by flow cytometry.

Sham transfection was used to provide the control cells for fluorescence microscopy. Cells were subjected to the transfection procedures described above, with the exception that no DNA was added.

Flow Cytometry

Flow cytometric cell sorting was performed using a Coulter EPICS Elite ESP Cytometer (Coulter Corporation, Miami, FL) equipped with an air-cooled Argon laser producing 488 nm light to excite fluorescein. Optical laser alignment calibration of the flow cytometer was performed using Coulter's DNA-Check EPICS alignment fluorosphere beads (Coulter Corporation). The interday coefficient of variation of the peak widths obtained with the fluorosphere bead standards was less than 2%, confirming consistent instrument performance. Cellular debris and doublets were eliminated by forward angled light scatter and 90o light scatter. Fluorescein light emission was reflected through a 550 nm dichroic long pass filter, which transmits all light with wavelengths of >550 nm. Light with wavelengths <550 nm are reflected off at a 90o angle and collected after being filtered by a 525 nm band pass filter. Background gating for positive cells containing internalized plasmid DNA did not exceed 1%. Viable cells were sorted at 1 droplet with a data rate of 5,000 events/sec for 3-4 hr using a 100-µm flow sense quartz tip with a frequency of 32 kHz and coincidence abort activated. Sorted cells were deflected through a 3000 V ceramic deflection sort plate and collected into sterile polypropylene tubes containing culture media maintained at 4oC using a temperature regulation module. Data files were stored in list mode format and extended analysis was performed using WinMDI data analysis software (Salk Institute, La Jolla, CA). The fractions of cells that had a fluorescence intensity significantly greater than the control value were considered positive for internalized plasmid DNA transfected, and were sorted and collected. The increase in the fraction of positive cells with time was determined from the flow histograms.

Viability of Sorted Cells

The viability of the transfected and sorted cells was determined by monitoring the ability of the cells to exclude the trypan blue dye (0.04% in 0.85% saline) and to maintain growth.

Bright Field and Laser Confocal Fluorescence Microscopy

Cells were resuspended in Antifade solution (Oncor, Gaithersburg, MD), at a density of >200,000 cells/ml. Five microliters of cell solution was placed on a glass slide, cover-slipped, and examined using bright field fluorescence microscopy. Microscopy was performed using an Axiovert 35 inverted microscope (Zeiss, Thornwood, NY) equipped with a 100× oil immersion lens (numerical aperture 1.25), Filter Set 09 (excitation 450-490 nm, beam splitter 510 nm, emission 520 nm), and mercury bulb UV illumination. Photomicrographs were taken using a camera directly mounted on the microscope.

Laser confocal fluorescence microscopy was used to ascertain the intracellular localization of the fluorescence label. We first removed the extracellular DNA by incubating the transfected cells with a DNAse I solution (HBSS, 5 mM MgCl2, 1% BSA, 1,000 units/ml DNAse I from Boehringer Mannheim) for 85 min. Cells that were transfected were fixed overnight in 2% wt/vol paraformaldehyde dissolved in phosphate-buffered saline (pH 7.4) at 4oC, and counterstained with 0.005% (wt/vol) Evans Blue (Sigma). Evans Blue stained the cell membrane, which enabled the visualization of the cell during confocal microscopy. In addition, the red color of Evans Blue could be easily distinguished from the green color exhibited by the FITC-labeled DNA. Five microliters of suspended cells (>200,000/ml) was placed on a glass slide and cover-slipped. Microscopy was performed using a Meridian ACAS 570 confocal microscope (Meridian Instruments, Okemos, MI) mounted on an Olympus IMT-2 inverted microscope with a 40× lens (numerical aperture 0.55). An argon laser emitting at 488 nm was used to excite the fluorescein-12-dUTP and Evans Blue fluorophores. The emitted light was split with a 575 short pass dichroic filter. The fluorescein-12-dUTP was detected with a 530/30 nm band pass filter, whereas the Evans Blue signal was detected with a 605 long pass filter. The cells were scanned in three dimensions.

RESULTS

DNA Fragmentation During Nick Translation

Results of agarose gel electrophoresis showed the presence of native plasmid DNA (~5 kb in size) and DNA fragments of ~2 kb and ~1 kb after 10 and 20 min incubation with the nick translation mixture. After incubation for 30, 60, and 90 min, only the ~2 kb and ~1 kb fragments but not the native DNA were present. The fluoresence label resided in the ~1 kb fragment, which was the predominant component at 90 min. Subsequent studies used the nick translated product obtained at 90 min.

Detection and Sorting of Fluorescein-12-dUTP-Labeled Cells

Figure 1A shows the relative fluorescence intensity in individual cells, as a function of the relative cell size and transfection duration. The background fluorescence signal of the non-transfected control cells was 1.2 units (Figure 1A). The fraction of cells that display fluorescence above the background level increased with duration of transfection, from 0.7 ± 0.6% (mean ± SD, n = 3) in nontransfected cells to 1.7 ± 0.8% after 5 min of transfection (n = 3, p = 0.08), 9.4 ± 6.3% at 1 hr (n = 4, p = 0.03), and 17.6 ± 9.7% after 3 hr (n = 4, p = 0.02). Likewise, the fluorescence intensity of individual cells increased with transfection duration, from 2 to 3 units after 5 min transfection to up to 30 units after 1 and 3 hr. To ascertain that there were no significant changes in cell size and/or cell death after transfection, the results in Figure 1A were also analyzed by plotting the forward scatter vs. side scatter (Figure 1B). A comparison of cells obtained before and after transfection shows no change in cell size distribution, as indicated by the similar forward scatter data and no change in granularity as indicated by the similar side scatter data. Because increases in granularity are associated with cell death/fragmentation and with cell clumping, no change in granularity suggests that the transfection procedures did not induce cell death. Figure 1C shows a shifting of the relative fluorescence of an increasing number of cells over time after transfection, indicating that either the fraction of cells with internalized DNA and/or the amount of the transfected DNA per cell increased with the duration of transfection.

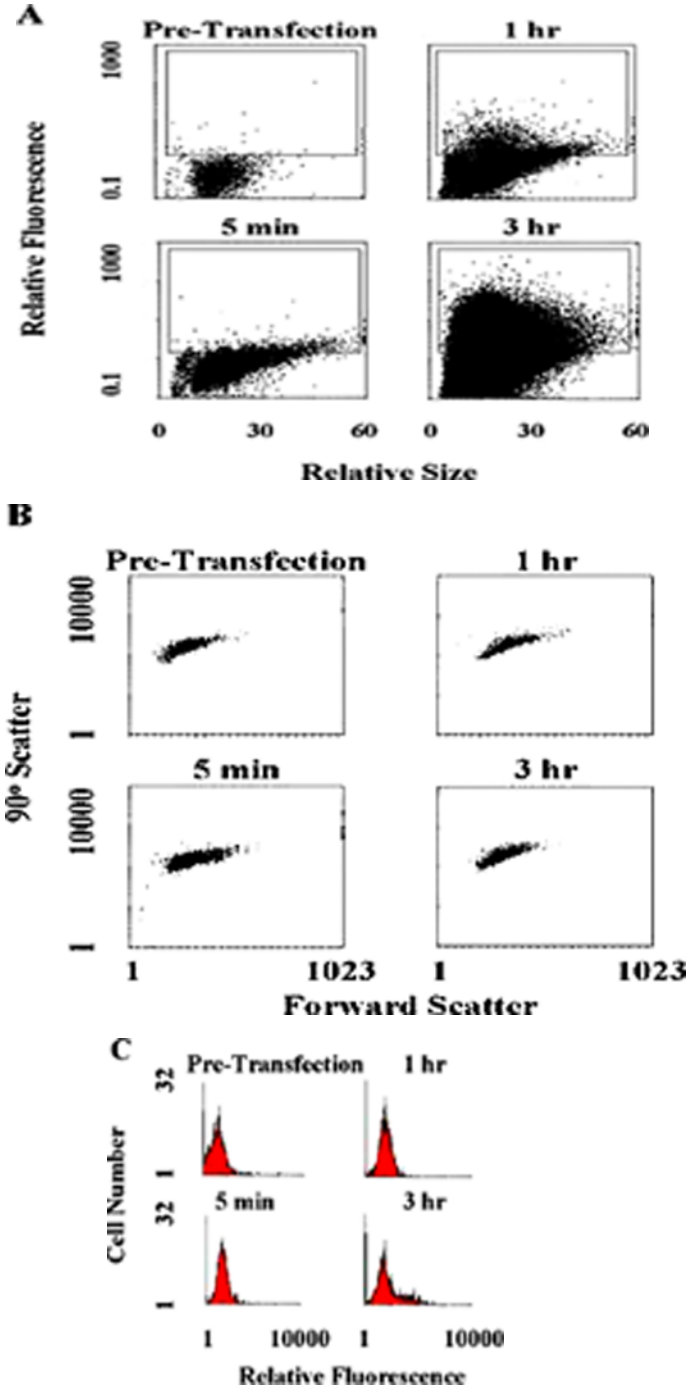

Figure 1. Flow cytometric analysis of transfected cells. Cells were transfected with pGL3 plasmid DNA pre-labeled with fluorescein-12-dUTP. A: Fluorescence signal of individual cells was expressed as a function of relative cell size and transfection duration. The box indicates the upper and lower limits of cell size and fluorescence intensity used for cell sorting. Cells with fluorescence intensity greater than the background level (i.e., >1.2 relative units) were considered positive for internalized plasmid DNA. The positive cells with a relative size of between 2 to 58 were sorted. B: Flow histograms showing side-scatter (90o scatter) versus forward scatter of cells with internalized plasmid DNA over time. C: Increase in the number of fluorescent cells over time.

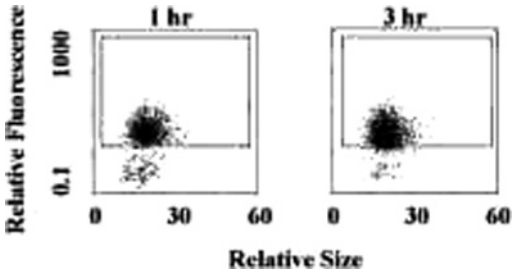

Figure 2. Purity of sorted cells. Cells transfected with fluorescein-12-dUTP-labeled plasmid DNA and sorted by flow cytometry were re-analyzed for their fluorescence signal. The fluorescence signal of individual cells was expressed as a function of cell size and transfection duration. Cells located inside the box were considered positive for internalized plasmid DNA. The purity was 94 and 96% for cells obtained after 1 and 3 hr transfection.

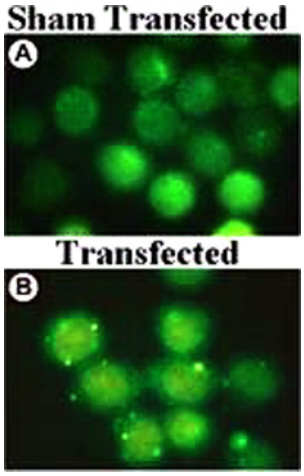

Figure 3. Fluorescence photomicrographs of cells containing internalized labeled plasmid. Cells were transfected with fluorescein-12-dUTP-labeled plasmid DNA. (A) Sham transfected cells. (B) Cells sorted as positive for internalized DNA. Note the punctated bright green fluorescein signal in panel B.

Cells that showed fluorescence intensity above the background level were sorted as positively labeled. Re-sorting of the positively labeled cells showed a 95% purity, ie, 95% of the sorted cells resided in the fluorescence-gated region (Figure 2). The remaining <5% cells, which showed a fluorescence signal of <1 unit, might have resulted from contamination and/or photobleaching of the fluorescein label by the laser during cell sorting.

Viability of Sorted Cells

More than 98% of the sorted cells, obtained immediately after flow cytometric cell sorting, excluded trypan blue dye. The same level of viability was observed in sham-transfected cells not subjected to sorting. Upon culturing, the sorted fluorescein-labeled cells increased with time, by 6.7 ± 0.5-fold after 120 hr (average ± range of two determinations). In comparison, the doubling time of untreated cells was ~24 hr. These data indicate the maintenance of cell viability and cell growth (albeit delayed) after transfection and sorting procedures.

Fluorescence Microscopy

We compared the fluorescence signal of cells that were sham-transfected (ie, processed for transfection without addition of plasmid DNA) with cells that were transfected with fluorescein-12-dUTP labeled plasmid DNA. The results of the bright field fluorescence microscopy showed the absence of fluorescence signal in the sham-transfected cells (Figure 3A), and the presence of the fluorescence label residing on the surface of and/or inside the cells with internalized plasmid DNA (Figure 3B).

To ascertain the intracellular location of the fluorescein label, cells were treated with DNAse I, which was found sufficient to completely degrade 100 µg of fluorescein-12-dUTP-labeled plasmid DNA complexed with Lipofectin. Confocal fluorescence microscopy was used to further visualize the fluorescence signal. Figure 4 shows serial images of the contents of the transfected cells, in successive 2-µm optical sections. The punctated fluorescence signal in the intracellular compartments confirms that the labeled DNA was located intracellularly and not extracellularly.

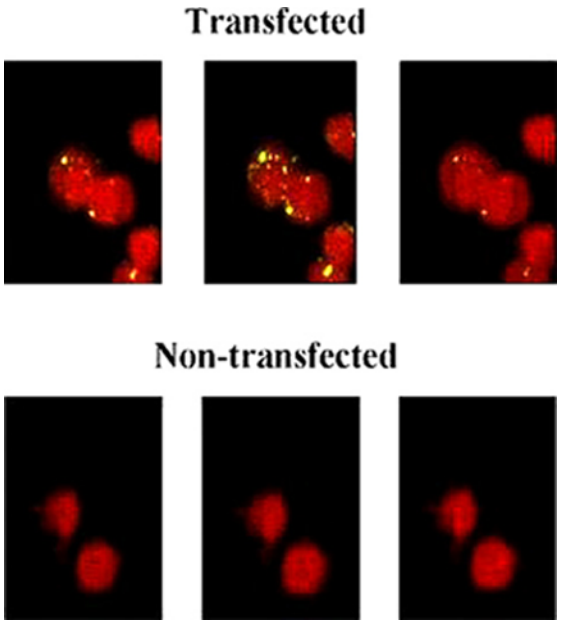

Figure 4. Fluorescence confocal photomicrographs of cells containing internalized labeled plasmid. Cells were transfected with fluorescein-12-dUTP-labeled plasmid DNA for 3 hr, treated with DNAse I to remove extracellular plasmid DNA, counterstained with Evans Blue (red color) and then analyzed by laser confocal microscopy. Serial optical sections (2 µm apart) are presented to confirm the intracellular localization of the fluorescein-12-dUTP- labeled DNA. The punctated green fluorescence in the positive cells confirm the successful transfer of DNA into the cell.

DISCUSSION

The present report describes a method for monitoring the transfer of DNA during transfection. The results show that the flow cytometric sorting detected cells that had internalized plasmid DNA as early as 1 hr after initiation of the transfection procedures as opposed to the 24 to 48 hr that is typically required for the measurement of the expression of a reporter gene. We further show that a combination of the flow cytometric sorting of the cells that have taken up plasmid DNA with laser confocal microscopic determination of the location of the transferred DNA (ie, extracellular vs. intracellular) enables the study of the kinetics of DNA movement. Other potential applications include studies of the efficiency of plasmid uptake, intracellular movement, localization in subcellular organelles, and processing of the internalized DNA. Because the present study was not designed to examine the cytoplasm-to-nucleus transfer of exogenous DNA, we did not examine the intracellular location of the transfected DNA. However, the central location of the fluorescent DNA in the successive 2-µm optical sections, together with the knowledge that over 80% of the volume of FaDu cells is represented by the nucleus, suggest that the transfected DNA is located in the nucleus. Further studies using markers to identify the nucleus, simultaneously with the detection of the labeled DNA using confocal microscopy, are needed to determine the intracellular location and the cytoplasm-to-nucleus transfer of the transfected DNA.

ACKNOWLEDGMENTS

The authors thank Joseph Trotter for the use of the WinMDI software that he developed. This work was partly supported by Research Grant R01CA77091 from the National Cancer Institute, NIH, DHHS. The Flow Cytometry Service was partly supported by the Cancer Center Support Grant P30CA16058 from the National Cancer Institute.

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